THE MUTAGENIC EFFECT OF THYMINE STARVATION ON *SALMONELLA TYPHIMURIUM*

by

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INTRODUCTION

A number of methods are now available to induce mutations in microorganisms (Freese, 1963). Since the discovery of the first induced mutation involving X-ray in Drosophila (Muller, 1928), the numbers and types of mutagenic agents have greatly increased. Almost all mutagens act, in one way or another, on the genetic material of the cell, deoxyribonucleic acid.

One class of mutagens acts by inhibiting the formation of nucleic acids and nucleic acid precursors (Freese, 1963). Cohen and Barner (1954), in their initial discovery of the effects of thymine starvation on bacteria, reported that in addition to causing cell death, DNA synthesis was inhibited. From the knowledge that certain mutagens act by inhibiting DNA synthesis it seemed possible that thymine starvation might be mutagenic. Thymine starvation was shown to be mutagenic for Escherichia coli strain 15T⁻ by reversion of existing auxotrophic markers (Coughlin and Adelberg, 1956; Kanazir, 1958).

The work discussed in the present thesis was undertaken to determine whether thymine starvation is capable of inducing "forward mutations" in Salmonella typhimurium strain LT2. A "forward mutation" is defined as one which results in the loss of some biochemical function. The exponential decrease in viable count of thymine starved cells served as a means

of selecting for induced mutants. The selective ability of thymine deficiency is based on the fact that the presence of a second auxotrophic marker in a thymineless bacterium allows such cells to remain viable in thymine free medium. Penicillin screening was also used to select for induced mutants.

Mutants induced by starvation were analyzed to determine the type of mutation produced. Analysis was accomplished by using various mutagens to revert induced markers (Freese, 1963). Hopefully, the reversion patterns would distinguish between "single site" and "multisite" mutations.

REVIEW OF LITERATURE

The phenomenon of thymineless death in E. coli strain 15T⁻ was first reported by Cohen and Barner in 1954. They observed that thymine deprivation of a thymine auxotroph resulted in the loss of the ability of starved cells to form colonies. Deprivation was also characterized by a 30 minute lag before the onset of exponential death. Barner and Cohen (1954) demonstrated that other biochemical mutants incubated in minimal medium did not die exponentially, nor did they multiply. Thus exponential death was shown to be unique for thymine auxotrophs deprived of thymine.

Thymineless death and unbalanced growth

Cohen and Barner (1954) observed that thymine starvation caused an immediate cessation of DNA synthesis but RNA synthesis continued normally. Starved cultures exhibited only a 15 to 20 per cent increment in DNA synthesis while RNA synthesis increased 50 to 75 per cent. Additional experimentation revealed that protein synthesis also continued normally during the initial stages of starvation (Cohen and Barner, 1955). This was evidenced by the formation of the inducible enzyme xylose isomerase in the presence of xylose but in the absence of exogenous thymine. Under conditions of continued RNA and protein synthesis, but retarded DNA synthesis, starved cells were observed to

undergo a marked increase in cell size (Cohen and Barner, 1954). They used the term "unbalanced growth" to describe this continued cytoplasmic activity, and it was suggested as the cause of cell death during thymine starvation.

Evidence to support the theory of unbalanced growth as the cause of thymineless death was obtained from experiments involving E. coli 15 under conditions of ultraviolet irradiation (Barner and Cohen, 1956). The initial killing action of ultraviolet irradiation was followed by a secondary reduction in viable cells resembling thymineless death. The conditions under which secondary death occurred were continued cytoplasmic synthesis, but inhibited DNA synthesis (Kanazir and Errera, 1956). Thus Barner and Cohen proposed that the cause of death in both cases was unbalanced growth.

The relationship of unbalanced growth to thymineless death and to secondary death due to ultraviolet irradiation was further investigated (Okagaki et al., 1960; Billen, 1959). By adding chloramphenicol (100ug/ml) at the onset of starvation, Okagaki et al. (1960) observed that thymineless death was inhibited. Chloramphenicol inhibited protein synthesis but allowed RNA synthesis to continue. Therefore it appeared that death might be due to an imbalance between protein and DNA synthesis. Okagaki et al. (1960) hypothesized that either the addition of thymine or chloramphenicol should prevent thymine deficient bacteria from dying. Experiments showed that the addition of thymine (2ug/ml) at

20 to 40 minutes after the start of thymine deficiency prevented thymineless death. However, the addition of chloramphenicol (100ug/ml) at the same time intervals did not prevent thymineless death even though protein synthesis was inhibited. These results indicated that thymineless death was not caused by unbalanced growth, but rather by some other events associated with thymine deficiency. Likewise, secondary death due to ultraviolet irradiation was a phenomenon separable from unbalanced growth. Although DNA synthesis was initially inhibited by UV inactivation, synthesis resumed once secondary death began. The loss of viability could not be attributed to unbalanced growth since death occurred while DNA, RNA, and protein synthesis took place.

Mechanism of thymineless death

Although unbalanced growth, as defined by Cohen and Barner (1954), could be ruled out as the cause of thymineless death, the true cause remained obscure. Cohen and Barner (1954) did show that the loss of viability was not initiated in the absence of thymine if glucose was omitted from the incubation medium. The results of Freifelder and Maaløe (1964) indicated that an energy source, such as glucose, was also necessary to sustain thymineless death. From these findings they concluded that it was possible to place several constraints on the mechanism of thymineless

death. Two hypotheses were proposed by Freifelder and Maaløe (1964) concerning the mechanism of the killing event. One hypothesis proposed that thymine starved cells accumulated some substance which at sufficiently high concentrations was lethal to affected cells. The lag period then represented the time necessary for accumulation of enough lethal substance to cause death. The alternative hypothesis was based on the theory that some chemical change took place in an essential cell component. One such change does not cause death; therefore the lag represented the time during which these chemical changes accumulated until the affected cells could no longer survive.

Soon after the discovery of thymineless death, Fuerst and Stent (1956) proposed that it was due to some type of nuclear damage. Evidence accumulated by many investigators suggested that the nuclear damage was in the form of DNA inactivation.

Pritchard and Lark (1964) reported that thymine starvation altered DNA replication in E. coli 15T⁻. Normally replication was observed to proceed from a point on the chromosome termed the "origin." During thymine starvation a second replication cycle was initiated prior to completion of the first replication cycle. Although two points of simultaneous DNA replication was abnormal, it was not thought to be the cause of thymineless death.

Maaløe and Hanawalt (1961) suggested that an irreparable mistake in attempted DNA synthesis was the cause of death. They reported that protein and/or RNA synthesis was necessary to initiate but not to sustain DNA synthesis. Once a DNA replication cycle was completed, another could not be initiated without preliminary protein and/or RNA synthesis. These findings helped to explain the earlier incorrect theories implicating unbalanced growth as the cause of thymineless death. Cytoplasmic synthesis appeared to be independent of the killing event since death occurred with equal efficiency in synthetic minimal medium and in the same medium enriched with 1.5 per cent casamino acids, even though mass synthesis was greater under the latter condition.

Mennigmann and Szybalski (1962) reported structural changes in DNA from thymine starved cells of E. coli 15T⁻. These structural changes appeared to be in the form of single stranded breaks in the DNA helicies. They theorized that such breaks would probably result in the collapse of its rigid structure. Evidence to support this model was given by the loss of viscosity, the loss of transforming ability, and the increased sensitivity to shear, of thymine starved DNA.

Gold and Hurwitz (1963) found that thymine starved DNA had a lesser tendency to become methylated than normal DNA. Whether or not these findings actually reflected alterations in starved DNA could not be determined.

Thymineless death was later described for a number of other bacteria; E. coli B_3 (Gallant and Suskind, 1962), Bacillus megaterium (Wachsman et al., 1964), and S. typhimurium (Eisenstark, unpublished). B. megaterium strain KM:T^- was observed to undergo thymineless death following a 60 minute lag period (Wachsman et al., 1964). The length of the lag period was notably longer than that reported for E. coli $15T^-$ by Cohen and Barner (1954). No evidence of cell lysis was observed even after 8 hours of thymine deprivation. Thymine starvation of E. coli B_3, as reported by Gallant and Suskind (1962), resulted in exponential cell death. Chloramphenicol treatment, used to inhibit only protein synthesis, did not prevent cell death in the absence of thymine. In contrast, cell death was prevented when RNA synthesis was inhibited. These findings contradict those previously reported by Okagaki et al. (1960) in E. coli $15T^-$. Gallant and Suskind (1962) suggested that thymineless death might be due to the synthesis of abnormal RNA; however, base composition and metabolic stability studies did not reveal any differences between RNA in normal or in thymine starved cells. They concluded that thymine starvation might somehow alter cellular DNA so that RNA synthesized off the DNA template would be defective, thus causing cell death.

Although the preceding evidence implicated actual structural damage to DNA as the cause of thymineless death, additional findings indicated that the killing event may

have been only indirectly associated with the state of the bacterial DNA. Melechen and Skaar (1960) reported the presence of a high number of P1b bacteriophage particles in thymine starved cultures of E. coli B_3 (P1b).

Similar observations were made by Korn and Weissbach (1962) with E. coli K12 $T^-(\lambda)$ and by Maisch and Wachsman (1964) with B. megaterium KM:T^-(L). Induction of P1b by starvation, or other means, was initiated by the uncoupling of prophage DNA from the bacterial chromosome, but in thymineless cultures, multiplication of the vegetative phage was prevented (Melechen and Skaar, 1962). The addition of thymine allowed phage induction to continue. Thymine deprivation apparently was associated with an early step in prophage induction, but it remains questionable whether induction was the cause of thymineless death. Evidence against death due to induction was that chloramphenicol treatment of E. coli B_3 (P1b) prevented phage induction but thymineless death was not inhibited (Melechen and Skaar, 1962). Furthermore, the rate of thymineless death in two strains of B. megaterium, KM:T^-(L) and KM:T^-, was similar even though one was lysogenic and the other was not lysogenic for any known phage (Wachsman et al., 1964). The possibility exists however, that strain KM:T^- actually was lysogenic for some unknown phage (Eisenstark, personal communication). Frampton and Brinkley (1965) induced phage-like particles in E. coli 15T^- using ultraviolet irradiation and 5-bromouracil

incorporation prior to irradiation. Induced lysates, when examined under the electron microscope, were found to contain both complete and incomplete phage particles. These induced particles were morphologically different from any previously known coliphages. None of the lysates were capable of plaque formation.

Various methods are available to induce bacteriophage and bacteriophage-like particles in bacterial cells. Mitomycin C treatment of B. subtilis induces bacteriophage-like particles incapable of plaque formation but capable of exerting a bacteriocidal action on sensitive cells (Subbaiah et al., 1965). Thymine starvation, as previously discussed, is known to induce certain bacteriophage, but induction of defective bacteriophage has not been definitely established (Mukai, 1960). Subbaiah et al. (1965) stated that thymineless death might be due to the induction of such defective bacteriophage.

An outward similarity exists between the mechanism of prophage induction and the induced synthesis of enzymes. Both systems could be dependent on the state of a repressor substance. Experiments were performed by Melechen (1964) to determine if prophage induction could be attributed to the destruction of a repressor by thymine starvation. Results indicated that induction was not due to derepression but rather to synthesis of abnormal DNA during starvation which caused dissociation of the prophage from the

cellular DNA.

Another observation in thymineless cells of E. coli K12(λ) was the production of deoxyribonuclease during induction of phage λ by thymine starvation (Korn and Weissbach, 1963). The presence of this exonuclease was also observed on infection of E. coli K12 with a lethal mutant of λ . No such exonuclease can be demonstrated before induction or infection.

Mennigmann (1964) proposed that thymineless death in E. coli 15T⁻ appeared to be related to the production of colicin, which was detectable only upon the readdition of thymine to starved cultures. Presumably thymine starvation altered the colicinogenic factor to an induced state where it could synthesize colicin as soon as thymine was available. Colicin action was characterized by the lysis of affected cells. Mennigmann (1964) concluded that the killing event in thymineless death did not take place in the starvation medium but rather on the nutrient agar used to assay for viable cells.

Selectivity of thymineless death

Barner and Cohen (1957) isolated four amino acid mutants of the thymineless bacterium E. coli 15T⁻. These mutants did not exhibit the characteristic death response when grown in medium lacking thymine and the required amino acid. Under these conditions thymineless death occurred,

but not to the extent that occurred in normal thymineless death. When the required amino acid was present in starvation media, the doubly auxotrophic mutants underwent exponential death. The amino acid requirement prevented protein and RNA synthesis in minimal medium. Assuming the observations of Maaløe and Hanawalt (1961) to be correct, RNA and/or protein synthesis inhibition allowed only limited DNA synthesis. Consequently thymineless death was almost entirely prevented. The amino acid requirement apparently only partially blocked protein synthesis since some loss of viability did occur. Chloramphenicol, for example, totally inhibited protein synthesis and death was completely prevented (Okagaki et al., 1960).

Thymineless death was used to select auxotrophic mutants of thymineless bacteria (Bauman and Davis, 1957; Wachsman and Hogg, 1964). The ability to select for polyauxotrophic mutants during starvation was based on the immunity of amino acid deficient cells to the killing process. Wachsman and Hogg (1964) exposed exponentially growing cultures of B. megaterium strain KM:T$^-$ to ultraviolet irradiation with the intent of inducing a second auxotrophic marker. Irradiated cells were then incubated in complete medium. Subsequent thymine starvation resulted in death to cells of strain KM:T$^-$, while cells containing a newly induced mutation remained viable. The experiments of Wachsman and Hogg (1964) also showed that the percentage of mutants among the

surviving cells increased with the time of starvation. In addition, they found that induced mutants had a higher survival rate the lower the population density. The percentage of mutants in the final population could also be increased by alternating periods of growth in the presence of amino acids and thymine starvation in the absence of amino acids.

Mutation by thymine starvation

Thymine deprivation was first shown to be mutagenic for E. coli 15T⁻ (Coughlin and Adelberg, 1956). A histidine requiring mutant of the thymineless strain was subjected to thymine starvation. The mutational event involved reverting the existing auxotrophic marker to histidine independence. Thymine starvation produced histidine independent cells at a rate higher than could be attributed to spontaneous reversion.

Weinberg and Latham (1956) reported the occurrence of substantial numbers of streptomycin resistant cells after 8.5 hours of thymine starvation in medium containing streptomycin. No difference in selectivity was observed between streptomycin resistant and streptomycin sensitive cells. Thus the effect of thymine starvation on deoxyribonucleic acid was attributed as the cause of the mutational event.

The mutagenic nature of thymine starvation was later verified by Kanazir (1958) in a uracil-less strain of

E. coli 15T⁻. The mutational event again involved reversion of the existing auxotrophic marker, in this case the uracil marker. Revertants were detected as early as 30 minutes after the onset of starvation. Mutation apparently also occurred after 30 minutes during exponential death, since uracil independent revertants decreased at a slower rate than uracil-less cells in synthetic medium containing uracil.

Much of the work involving thymine deprivation was aimed at understanding the mechanism by which it caused cell death. Little investigation has been done concerning the mutagenic nature of thymine starvation; consequently the cause of mutagenicity is unknown. Dunn and Smith (1958), however, reported an increase in the base analog 6-methyl aminopurine in thymine starved cells. If this incorporation altered the nucleotide base sequence, mutation might have occurred. No evidence was obtained to prove that such an alteration actually took place. Kanazir (1958) suggested that mutation by thymine starvation involved a minor structural alteration of the bacterial DNA. Furthermore, he proposed that mutation and death might be different degrees of expression of the same phenomenon.

Many investigators are continuing their efforts to determine the mechanism of thymineless death and its mutagenicity.

MATERIALS AND METHODS

Bacteria and bacteriophage

All studies were conducted with mutants of Salmonella typhimurium strain LT2. Mutants were; KSU# 3507 (met try thy), KSU# 2213 (thy), and KSU# 2290 (arg thy). Bacteriophage P22 was used to transduce prototrophic markers of donor bacteria to recipients with induced auxotrophic markers. Bacteriophage involved in phage typing experiments were; P22, CDC, ØX174, F_2HfrC, MS_2, Mu1, cih+ SH45 Abony, p221-3c, m12, m13, and Sal. Specific spec.

Media

Cells were starved in minimal broth medium devoid of thymine. The minimal medium consisted of 10 ml distilled water, 10 ml minimal salt solution, and 0.2 ml of 40 per cent glucose per 20 ml. All inorganic salt components of the minimal salt solution were identical to those of the minimal agar discussed below with the exception that the salt solution contained no agar.

Plating for nutritional tests and for isolation of new mutants was done on minimal and nutrient agar. The minimal agar contained per liter of distilled water: K_2HPO_4 (21gm), KH_2PO_4 (9gm), $(NH_4)_2SO_4$ (2gm), sodium citrate (.94 gm), $MgSO_4$ (0.1gm), and Bacto-agar (15gm). The nutrient agar contained per liter of distilled water: Bacto-peptone

(10gm), beef extract (3gm), NaCl (5gm), and Bacto-agar (15gm). Reversion tests and penicillin screening were performed on enriched medium. Composition of this medium was minimal agar supplemented with 1/40 nutrient broth.

For maximum thymineless death to occur it was necessary to supplement the starvation medium with various amino acids depending on the mutant involved. All amino acids were supplemented at 15 ug/ml which was in excess of that normally required for cell growth. Thymidine and thymine have been shown to be utilized with equal efficiency (Cohen and Barner, 1954; Wachsman et al., 1964). Where necessary, thymidine was used to supplement the growth medium.

Five amino acid pools, one purine-pyrimidine pool, and one vitamin pool were used to aid in the identification of induced markers. All pool constituents were present at a concentration of 2mg/ml in distilled water.

All dilutions were performed in minimal salt solution.

Mutagens

The following mutagens were used to attempt to revert induced mutations: Diethyl sulfate used full strength (Fischer Scientific Co.), N-methyl-N'-nitro-N-nitrosoguanidine used as crystals (Aldrich Chemical Co.), 2-amino purine nitrate used as saturated solution (Sigma Chemical Co.), 5-bromodeoxyuridine used as saturated solution (National Biochemicals Co.), beta-propriolactone used as saturated

solution (Eastman Organic Chemicals), and proflavin used either as crystals or saturated solution (Allied Chemical Co.).

Mutagens used in the present study were known to be effective in reverting "single site" alterations. Diethyl sulfate (DES) and N-methyl-N'-nitro-N-nitrosoguanidine (NG) produce both transitional and non-transitional base pair changes (Freese, 1963; Eisenstark et al., 1965). Similar alterations occur with the alkylating agent beta-propriolactone (BPL) (Demerec, 1953). The base analogs 2-aminopurine nitrate (AP) and 5-bromodeoxyuridine (BDU) revert auxotrophic mutations by causing only transitional changes (Strelzoff, 1962; Rudner, 1960). The mode of action of proflavin (PRO) is not entirely clear. Freese (1959) proposed that proflavin produced transversion type mutations, while Brenner et al. (1961) suggested that proflavin acts by removing or inserting single base pairs. A thorough discussion of mutagens and their mode of action can be found in Freese (1963).

Inducing mutants by thymine starvation

Parental cells were inoculated into minimal medium supplemented with the required amino acids and thymidine, and incubated with shaking overnight resulting in a titer of approximately 10^9 cells/ml. The culture was centrifuged and washed twice to remove residual thymidine. For the

first starvation, washed cells were diluted 10^3 into minimal medium devoid of thymine, but containing the required amino acids. The length of the starvation period varied between five to seven hours, and the titer after starvation was approximately 5×10^4 cells/ml. At this point in the procedure a small percentage of the surviving cells were expected to contain an additional auxotrophic marker due to the mutagenic action of thymine deficiency.

Selecting mutants induced by thymine starvation

The induced mutant cells were present in the surviving population in such low numbers that it was necessary to select for them. This selection was accomplished by employing a second thymine starvation. The use of this technique required an intermediate growth phase between the first and second starvations. The intermediary growth phase permitted both parental and induced mutants to multiply. A small number of cells that survived the first starvation were incubated in nutrient broth at 37° C for eight hours. These cells were then centrifuged, washed twice and diluted 10^2 into properly supplemented minimal medium for the second starvation. Time of the second starvation varied depending on the final titer desired. The use of the second starvation for selecting induced mutants was based on the findings of Barner and Cohen (1957), who reported that only actively growing cells succumb to thymineless death. The additional

induced auxotrophic marker prevented active biosynthesis in mutant cells. After starvation the viable cell population could be expected to contain an increased proportion of newly induced mutants. Evidence to support the efficiency of this procedure for selecting induced mutants of S. typhimurium LT2 was obtained by conducting starvation experiments and plotting resulting death curves.

Isolation of the selected mutants was accomplished by the replica plating technique (Lederberg and Lederberg, 1952). Samples from secondary starved cultures were spread on nutrient agar and incubated overnight. Resulting colonies were transferred by velveteen printing onto supplemented minimal agar plates. Colonies that arose on the nutrient plates, but not on the partially supplemented minimal plates, consisted of cells containing an additional mutation.

A second method of selecting induced mutants involved the use of the penicillin screening technique (Davis, 1948). A sample of cells that survived the first starvation were inoculated into nutrient broth for the intermediate growth phase. Penicillin screening was conducted on a portion of the intermediate growth culture. The concentration of penicillin in the screening medium was 75 units/ml, and the treatment time was 18-20 hours (Goodgal et al., 1964). After completion of the penicillin treatment, cells were plated on "double-enriched medium" which consisted of minimal agar supplemented with 1/40 nutrient broth. The medium

also contained amino acids required by the parental organism. Consequently, parental type cells formed normal colonies whereas cells possessing an additional mutation formed small colonies due to the limited amount of nutrient broth present in the plating medium.

Identification of auxotrophs

The identification of induced markers was accomplished by spreading 0.1 ml of an overnight culture on a properly supplemented minimal plate. Vitamin, purine-pyrimidine, and amino acid pools were spot tested to reveal which pool stimulated growth. By individually checking each component of the implicated pool, the biochemical requirement of the mutant was determined.

Nutritional test

The nutritional test was used to analyze all new mutants to be certain they also contained the markers of the parental organism. Approximately 10^8 cells were placed on minimal agar plates supplemented with combinations of the requirements, but devoid of one requirement. The missing supplement was then tested by adding crystals to a small spot on the appropriate plate. A growth response in the supplemented area on each plate indicated that the cells possessed parental markers plus the newly induced marker. The nutritional test prevented the isolation of contaminants containing

mutations. The test also gave information concerning the stability of induced markers.

Phage typing

The phage typing pattern of all mutants was used to ensure selecting only mutants of the parental organism, S. typhimurium LT2. Typing was performed on nutrient agar plates, and bacteriophage were applied by sterile applicator sticks to plates seeded with the test host. Results were scored as either the presence or absence of a clear zone after six hours incubation.

Reversion of induced mutations

Each mutant, induced by thymine starvation, was checked for revertibility with a number of chemical mutagens. From an overnight culture, 0.1 ml was spread on a minimal agar plate containing all biochemical requirements except the requirement due to the induced mutation. Mutagens were applied by spotting on the agar surface. All plates were incubated 48 hours before results were recorded, and tests with questionable results were repeated on enriched minimal medium.

Whenever an experiment was conducted involving reversion of auxotrophic markers by chemical mutagenesis, the problem arose as to whether or not the revertants represented the restoration of the original nucleotide sequence.

Revertant colonies could have resulted that were not true reversions. Methods by which this could have occurred have been cited by Eisenstark and Rosner (1964).

The techniques employed in the present paper did not allow one to distinguish between the various types of reversion. One exception, however, was the fact that some suppressor mutations could be distinguished from true revertants on the basis of colony size. The suppressor revertants consistantly formed smaller colonies on minimal medium due probably to incomplete enzyme activity. With this detectable exception in mind, the assumption was made that revertant colonies represented the restoration of the original nucleotide sequence.

Terminology

A number of abbreviations appear in the tables and text of this thesis. Abbreviations for mutagens were; diethyl sulfate (DES), N-methyl-N'-nitro-N-nitrosoguanidine (NG), 2-aminopurine nitrate (AP), 5-bromodeoxyuridine (BDU), beta-propriolactone (BPL), and proflavin (PRO). Abbreviations for biochemical mutants were; arginine (arg), aromatic amino acids (aro), cysteine (cys), histidine (his), isoleucine-valine (ilv), isoleucine (ile), leucine (leu), lysine (lys), methionine (met), phenylalanine (phe), purine (pur), threonine (thr), thymine (thy), tryptophan (try), and complex mutants (comp).

The following terms are defined to ensure correct interpretation. A "site" is defined as a single nucleotide base pair. "Single site mutations" are mutations resulting from the alteration of one nucleotide pair either as a transitional or nontransitional change. A "multisite mutation" is defined as a mutation involving several adjacent "sites," and it may extend over more than one gene (Hartman, 1962).

Theoretically, all "single site" alterations can be reverted both chemically and spontaneously. "Multisite" alterations, on the other hand, usually cannot be reverted.

Thymineless death curves

Cells from the thymineless organism S. typhimurium LT2 KSU# 2290 (arg thy) were starved of thymine in minimal broth medium to determine the extent of cell death during thymine deprivation. An overnight culture was centrifuged, washed twice, and diluted 10^2 into the starvation medium. The starvation medium contained arginine but lacked thymine. A control experiment was performed in which cells were starved only of arginine. In both cases, cell counts were made by removing samples at 30 minute intervals, diluting in minimal salts, and plating on nutrient agar.

Additional death curve experiments were conducted to determine the selective ability of thymine starvation. Doubly auxotrophic mutant cells of KSU# 2290 (arg thy) were

starved in medium lacking both arginine and thymine. The arginine marker theoretically represented a mutation induced by thymine starvation. Viable cell counts were taken, as described above, to determine the effect of the additional auxotrophic marker on thymineless death. Normal thymineless death served as a control.

RESULTS

Induced mutants

Forty-eight mutants were isolated as a result of seventeen starvation experiments with no more than five mutants isolated from a single experiment. Eight attempts were made in which no mutants were isolated. Six of these attempts involved KSU# 3507 (met try thy) as the parent organism.

After a number of mutants had been isolated, KSU# 3507 (met try thy) appeared to develop an immunity to thymineless death. Cells were observed to undergo a slight increase in titer after starvation, rather than the expected exponential decrease. Attempted corrective measures included washing cells thoroughly, checking media and supplements for contaminants, and single colony isolation of KSU# 3507 (met try thy), all of which were unsuccessful. KSU# 2213 (thy) was then thymine starved to re-check the mutant induction procedure. Two mutants were recovered on the first attempt.

KSU# 2290 (arg thy) replaced KSU# 3507 (met try thy) as the parental organism into which new mutations were placed. Seventeen mutants were isolated from KSU# 3507 (met try thy), two from KSU# 2213 (thy), and twenty-nine from KSU# 2290 (arg thy). Tables I and II list all mutants and their biochemical requirements.

Most of the induced mutants were satisfied by a single biochemical requirement. Exceptions included ilv 2406,

ilv 2414, ilv 2428, and ilv 2439 which required both isoleucine and valine for growth, and pur 2442 which required both adenine and thiamine for growth. The requirements for the complex mutant 2421 were not determined. The double requirement of isoleucine-valine mutants and adenine-thiamine mutants was shown to be the result of single mutational events (Wagner and Berquist, 1960; Yura, 1956). Therefore all mutations induced by thymine starvation, including those with multiple requirements, could have resulted from a single mutation. Reversion studies were then performed to determine if induced mutations were "single site" or "multisite" alterations.

Reversion of induced mutations

The reversion patterns of induced mutants are recorded in Tables I and II. All mutants reverted by 2-aminopurine nitrate (AP), 5-bromodeoxyuridine (BDU), beta-propriolactone (BPL), and proflavin (PRO) were also reverted by diethyl sulfate (DES) and N-methyl-N'-nitro-N-nitrosoguanidine (NG).

Twelve of the forty-eight mutants could not be reverted by the action of any chemical mutagen. The non-revertible mutants were; his 2407, ilv 2406, ile 2417, leu 2435, aro 2437, cys 2441, cys 2450, his 2451, ilv 2428, lys 2422, try 2445, and comp 2421. The remaining thirty-six mutants were reverted by both diethyl sulfate (DES) and N-methyl-N'-nitro-N-nitrosoguanidine (NG).

"Mutagen stable" mutations

Certain of the non-revertible mutants were observed to revert spontaneously even though they could not be reverted chemically. Demerec (1953) used the term "mutagen stable" to describe such mutants in Escherichia coli, and Hartman (1956) later reported the occurrence of mutagen stable mutants in Salmonella typhimurium. Mutagen stable mutants induced by thymine starvation were; ilv 2406, ile 2417, leu 2435, and aro 2437.

Transduction of induced mutants to prototrophy

Most of the thymine starvation induced mutants were transduced to prototrophy by "wild type" P22 bacteriophage. Mutants unable to be transduced were; ilv 2406, ile 2417, vit 2410, cys 2450, lys 2422, and comp 2421. The latter three mutants were suspected to involve "multisite" genetic changes, since they could not be chemically reverted. Two of the non-transducible mutations, ilv 2406 and ile 2417, were classified as mutagen stable mutants. The only mutant that was chemically revertible, but non-transducible, was vit 2410.

Mutants exhibiting "multisite" characteristics

On the basis of chemical mutagen reversion data, eight of the induced mutants appeared to represent genetic

alterations greater than single base pair changes. Excluding all mutagen stable mutants, the possible "multisite" mutants were; his 2407, cys 2441, cys 2450, his 2451, ilv 2428, lys 2422, try 2445, and comp 2421. These mutants did not revert spontaneously nor were they reverted by any chemical mutagens.

Transduction experiments involving these suspected "multisite" mutants showed that only cys 2450, lys 2422, and comp 2421 were not transduced to prototrophy by "wild type" P22 bacteriophage.

Thymineless death in Salmonella typhimurium LT2

Thymine deprivation of KSU# 2290 (arg thy) resulted in exponential death of starved cells. Starved cultures were incubated shaking at 37C, and a 60 minute lag was observed before the onset of exponential death. Viable cells decreased at a rate of one decade every three hours until approximately ten hours had elapsed, leaving a small fraction of surviving cells. The death curve and control appear in Figure 1.

The efficiency of thymine starvation as a selective device is shown in Figure 2. Cells of KSU# 2290 (arg thy) were incubated shaking at 37C in medium devoid of both arginine and thymine. The effect of an additional auxotrophic marker on thymineless death is illustrated by the upper curve. Cells possessing the additional mutation did

not undergo thymineless death when incubated in medium devoid of both metabolites. The lower curve represents the control, normal thymineless death.

TABLE I

Reversion patterns and nutritional requirements of mutants induced by thymine starvation in KSU# 3507 (met try thy), and KSU# 2213 (thy).

Mutant	Reversions (mutagens)							Phage transductions	Nutritional requirements
	Spon	DES	NG	AP	BDU	BPL	PRO		
arg 2408	+	+	+	-	-	-	-	+	arginine
arg 2415	+	+	+	-	-	-	-	-	arginine
aro 2409	+	+	+	+	-	-	-	+	tyrosine or phenylalanine
his 2407	-	-	-	-	-	-	-	+	histidine
his 2412	+	+sm	+sm	-	-	-	-	+	histidine
ilv 2406	+	-	-	-	-	-	-	-	isoleucine and valine
ilv 2414	+	+	+	-	-	-	-	+	isoleucine and valine
ile 2401	+	+	+	+	-	-	-	+	isoleucine
ile 2402	+	+	+	-	-	-	-	+	isoleucine
ile 2417	+	-	-	-	-	-	-	-	isoleucine
leu 2404	+	+	+	+	-	+sm	-	+	leucine
leu 2411	+	+	+	+	-	-	-	+	leucine
leu 2435*	+	-	-	-	-	-	-	+	leucine
phe 2413	+	+	+	-	-	-	-	+	phenylalanine
phe 2436*	+	+	+	-	-	-	-	+	phenylalanine
pur 2403	+	+	+	+	+	+sm	-	+	adenine
pur 2405	+	+	+	+	-	-	-	+	adenine and thiamine
thr 2400	+	+	+	-	-	+sm	-	+	threonine
vit 2410	+	+	+	-	-	-	+	-	vitamin

*Denotes a mutant derived from KSU# 2213 (thy)
sm-revertant colonies were small (possibly suppressor mutations)

TABLE II

Reversion patterns and nutritional requirements of mutants induced by thymine starvation in KSU# 2290 (arg thy).

Mutant	Reversions (mutagens)							Phage transductions	Nutritional requirements
	Spon	DES	NG	AP	BDU	BPL	PRO		
aro 2437	+	-	-	-	-	-	-	+	tyrosine or phenylalanine
cys 2425	+	+	+	+	-	-	-	+	cysteine
cys 2441	-	-	-	-	-	-	-	+	cysteine
cys 2446	+	+	+	-	-	-	-	+	cysteine
cys 2450	-	-	-	-	-	-	-	+	cysteine
cys 2452	+	+	+	-	-	-	-	+	cysteine
his 2448	+	+	+	-	-	-	-	+	histidine
his 2451	-	-	-	-	-	-	-	+	histidine
ilv 2428	-	-	-	-	-	-	-	+	isoleucine and valine
ilv 2439	+	+sm	+sm	-	-	-	-	-	isoleucine and valine
ile 2432	+	+	+	-	-	-	-	+	isoleucine and valine
leu 2427	+	+	+	-	-	-	-	+	leucine
leu 2429	+	+	+	+	-	-	-	+	leucine
leu 2438	+	+	+	-	-	-	-	+	leucine
leu 2449	+	+	+	-	-	-	-	+	leucine
met 2420	+	+	+	+	-	-	-	+	methionine
met 2424	+	+	+	-	-	-	-	+	methionine
met 2444	+	+	+	-	-	-	-	+	methionine
met 2447	+	+	+	-	-	-	-	+	methionine
lys 2422	-	-	-	-	-	-	-	-	lysine
phe 2423	+	+	+	+	-	-	-	+	phenylalanine

TABLE II
(Continued)

Mutant	Reversions (mutagens)							Phage transductions	Nutritional requirements
	Spon	DES	NG	AP	BDU	BPL	PRO		
phe 2426	+	+	+	+	+	-	-	+	phenylalanine
phe 2431	+	+	+	-	-	-	-	+	phenylalanine
pur 2442	+	+	+	-	-	-	-	+	adenine
pur 2443	+	+	+	-	-	-	-	+	adenine and thiamine
thr 2433	+	+	+	-	-	-	-	+	threonine
thr 2440	+	+	+	-	-	+sm	-	+	threonine
thr 2453	+	+	+	-	-	-	-	+	threonine
try 2445	-	-	-	-	-	-	-	+	tryptophan
comp 2421	-	-	-	-	-	-	-	-	not determined

Mutants 2445 through 2453 were selected by penicillin screening. All other mutants were selected by secondary thymine starvation and isolated by replica plating.

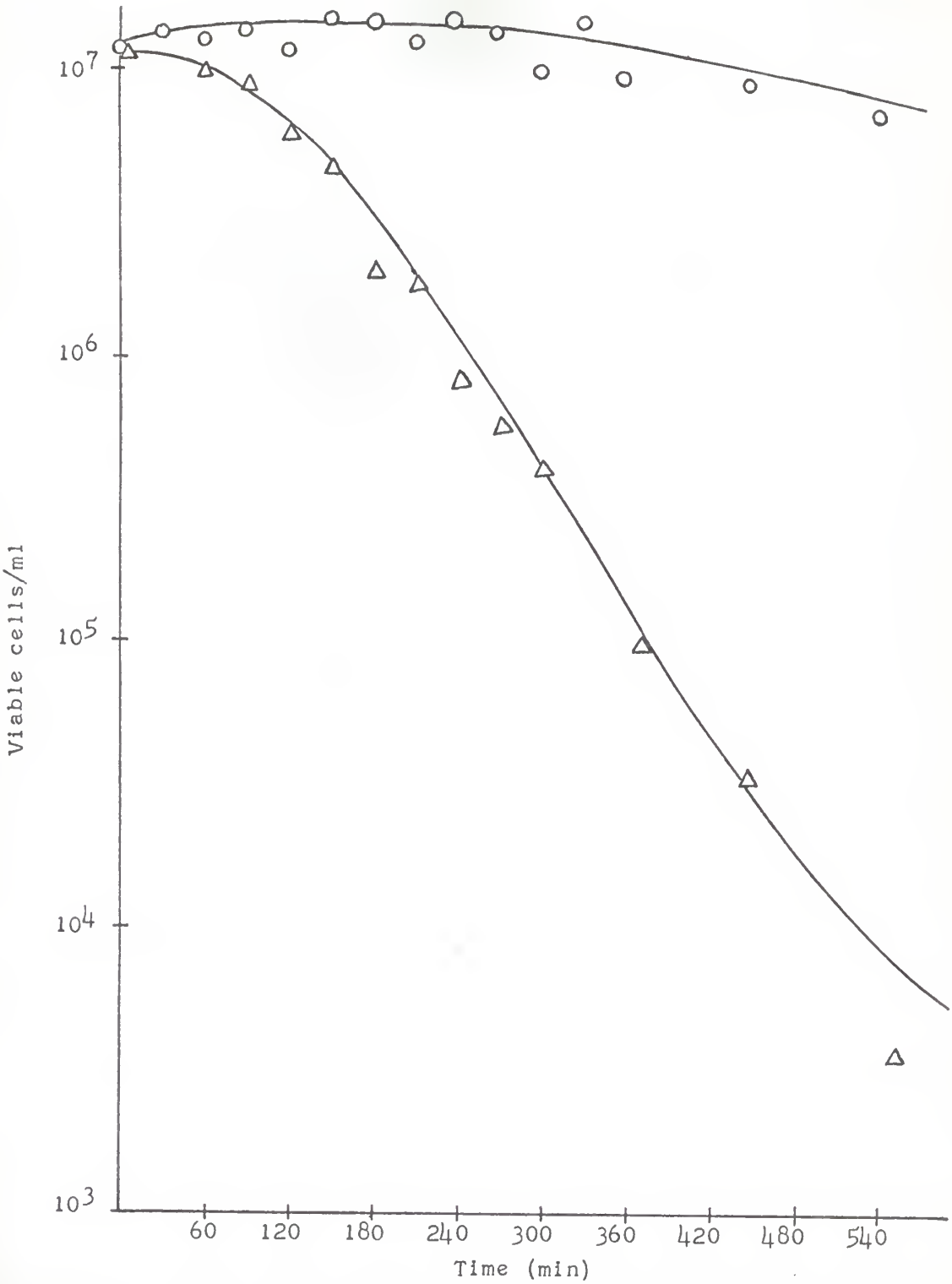

Fig. 1. Thymineless death in Salmonella typhimurium KSU# 2290 (arg thy). Δ - Survival curve of thymine starved cells, thymineless death. O - Survival curve of arginine starved cells.

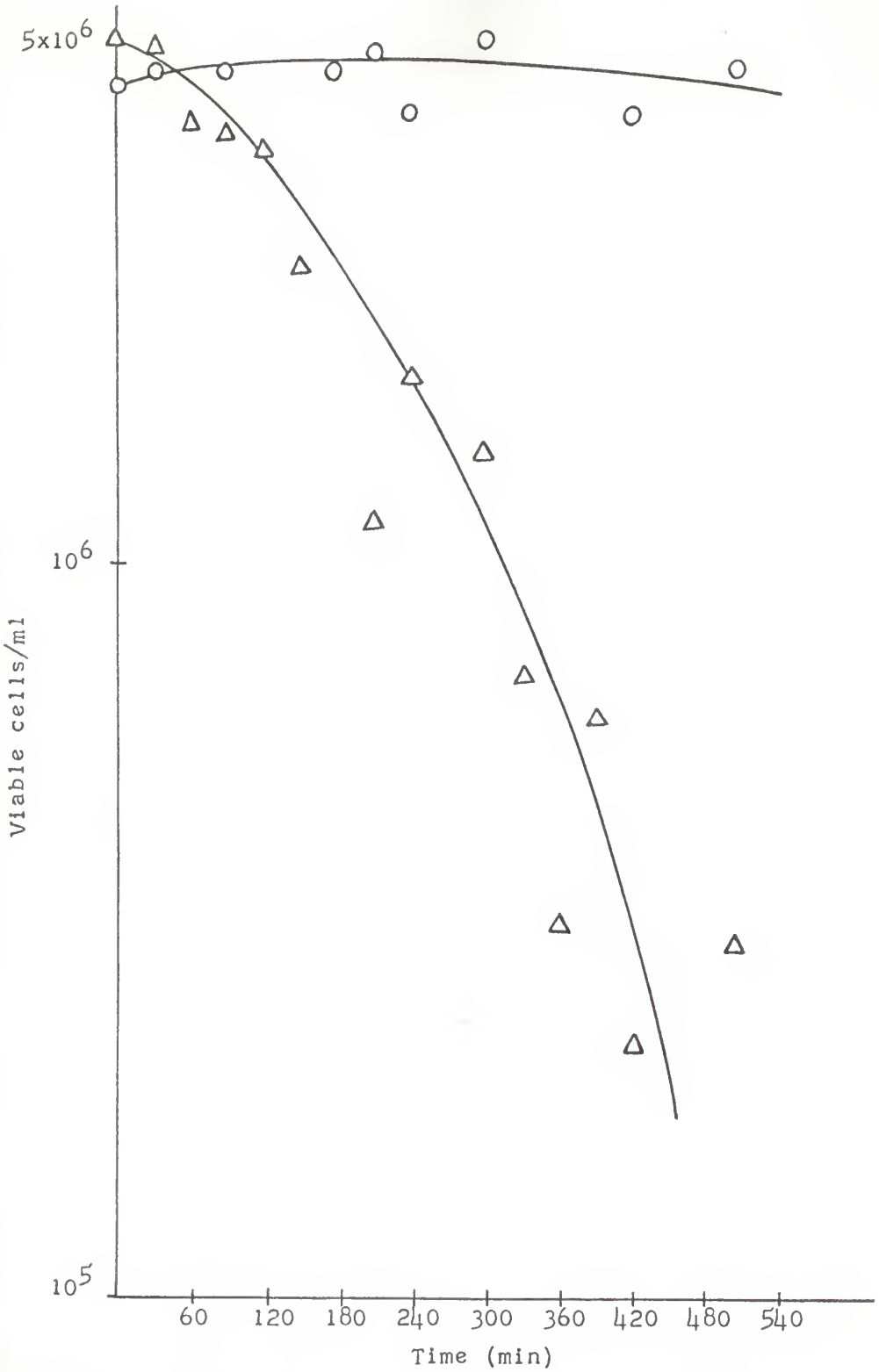

Fig. 2. The selective ability of thymine deprivation in S. typhimurium KSU# 2290 (arg thy). O - Survival curve of arginine and thymine starved cells. △ - Thymineless death.

DISCUSSION

Thymine deprivation was shown to induce forward mutations in Salmonella typhimurium LT2. Forty-eight mutants were isolated.

No attempt was made to calculate a numerical value for the forward mutation rate. Mutations occurred in the starvation medium with a frequency too low to detect on primary isolation; therefore starved cells were grown in nutrient broth before selecting for induced mutants. The low number of mutants was not unexpected since thymine starved cells were not dividing. Therefore, they would probably not be as susceptible to mutation as would dividing cells (Ryan, 1955).

Due to the low number of mutants upon thymine deprivation, it was necessary to establish that new mutants were actually induced by starvation and not induced spontaneously. Various precautionary measures were taken: (1) Parental organisms were chosen which were stable with respect to their forward mutation rate; and (2) in preparation for starvation experiments, single clones were inoculated into minimal medium supplemented with only the metabolites required by the parental strain. Any spontaneously induced mutants present in the initial population were restricted in their ability to multiply since an essential requirement was absent from the incubation medium.

Spontaneous mutants were most likely to appear during the intermediate growth phase between starvation periods. In order to minimize their occurrence, intermediate cultures were allowed to reach a titer of only 10^8 cells/ml, a population too low for many spontaneous mutants to arise. In addition, control experiments were conducted involving penicillin screening of intermediate growth phase cultures which had previously been starved of a requirement other than thymine. No mutants were recovered under these circumstances.

Eight of the thymine starvation induced mutants appeared to involve multisite alterations. The fact that they were not reverted by the action of any chemical mutagen and were not reverted spontaneously, provides evidence to support their "multisite" nature. Four other mutants were also non-revertible by chemical mutagens. However, they did revert spontaneously. Kirchner (1960) suggested that these "mutagen stable" mutants may represent transversional "single-site" alterations unaffected by mutations or they may represent alterations greater than "single site" changes. The remaining thirty-six mutants were concluded to be "single site" mutations, since they were all reverted by at least two of the mutagens tested.

Cells deprived of thymine underwent exponential death. The decrease of one decade every three hours varied considerably from the decrease of one decade per hour observed in other organisms (Cohen and Barner, 1954; Wachsman *et al.*,

1964). Of additional importance, it should also be noted that thymine starvation was shown to be an effective means of selecting induced auxotrophs. The additional mutation provided thymineless bacteria immunity from thymineless death (Barner and Cohen, 1957).

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THE MUTAGENIC EFFECT OF THYMINE STARVATION ON *SALMONELLA TYPHIMURIUM*

by

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ABSTRACT

A thymine auxotroph of Salmonella typhimurium was subjected to thymine deprivation and the starved cells were observed to undergo exponential death at a decrease of one decade every three hours. Analysis of starved cultures resulted in the recovery of cells possessing additional auxotrophic markers. Induced mutants were selected by secondary thymine starvation or penicillin screening, during which the parental type cells lost viability, whereas induced mutants were immune to the killing event. Forty-eight mutants were isolated.

Chemical mutagens, which act by altering single nucleotide base pairs, were used to determine the revertibility of induced mutants. The presence of revertant colonies due to spontaneous reversion or reversion by mutagen treatment signified the mutation initially represented a "single site" alteration, whereas the absence of revertants indicated a "multisite" change.

Thymine starvation of S. typhimurium LT_2 was found to induce both "single site" and "multisite" mutations. Thirty-six of the induced mutants were classified as "single site" since they were reverted spontaneously and by mutagens. Eight of the mutants were non-revertible by either method and therefore were assumed to represent "multisite" mutations. Four of the isolated mutants reverted spontaneously

but were immune to the various mutagens. It was not possible to establish the genetic nature of these mutants.

All apparent "single site" mutants were reverted by the mutagens diethyl sulfate (DES) and nitrosoguanidine (NG). The base analog 2-aminopurine nitrate (AP) reverted ten of the "single site" mutants while the other mutagens, including the base analog 5-bromodeoxyuridine (BDU), were ineffective in inducing revertants.

Although thymine starvation has been shown to induce "forward mutations," the mechanism of mutation is not known. Whatever the mechanism it must apparently be capable of inducing both "single site" and "multisite" mutations.